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THE MURINE LYMPHOCYTE RECEPTOR FOR IgE

III. Use of Chemical Cross-linking Reagents to Further Characterize the B Lymphocyte Fc$_\epsilon$ Receptor[1]

WILLIAM T. LEE[2] AND DANIEL H. CONRAD

From the Subdepartment of Immunology, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205

Cross-linking reagents were used to further characterize the murine B cell receptor for the Fc portion of IgE (Fc$_\epsilon$R) and compare this receptor to the well-characterized high-affinity Fc$_\epsilon$R on rat basophilic leukemia (RBL) cells. The disulfide cleavable and noncleavable reagents 3,3′-dithio*bis*(sulfosuccinimidyl) propionate (DTSSP) and *bis*(sulfosuccinimidyl) suberate (BS[3]) were used. With these reagents, efficient cross-linking of the α component of the high-affinity RBL Fc$_\epsilon$R to the membrane-buried β and γ components occurred only if the membrane was solubilized before the cross-linking reaction. In studies with purified murine B cells, IgE could be cross-linked to the Fc$_\epsilon$R on intact cells with either DTSSP or BS[3]. Under the same conditions, up to 10% of the B cell surface immunoglobulin (sIg) (both IgM and IgD) was also found to cross-link to a portion of the IgE/Fc$_\epsilon$R complex, suggesting that on the intact murine B cell the Fc$_\epsilon$R is frequently in close association with sIg. The B cell Fc$_\epsilon$R was also examined for the presence of receptor-associated proteins. Under conditions where the high-affinity RBL Fc$_\epsilon$R was substantially cross-linked to the α, β, γ complex, no evidence was seen for similar cross-linking of the B cell Fc$_\epsilon$R. Cross-linking experiments on affinity-purified Fc$_\epsilon$R preparations also gave no evidence for receptor-associated proteins with the B cell Fc$_\epsilon$R, although evidence for receptor-receptor association was seen. Thus, these data further support the concept that there may be little relationship between the high-affinity mast cell/basophil Fc$_\epsilon$R and the low-affinity lymphocyte Fc$_\epsilon$R.

Low-affinity receptors for the Fc region of IgE (Fc$_\epsilon$R)[3] were initially described in the human system by Gonzalez-Molina and Spiegelberg (1). Further investigation demonstrated the presence of the low-affinity Fc$_\epsilon$R on subpopulations of B cells, T cells, and monocyte/macrophages in various species (reviewed in Reference 2). The finding that the murine B cell Fc$_\epsilon$R has a relatively high affinity for IgE ($K_a = 10^8\ M^{-1}$) (3) and that its expression is enhanced by prior parasite infection (4) made the murine model attractive for further studies. Recently, the murine B cell Fc$_\epsilon$R has begun to be biochemically characterized. We have showed that the surface-exposed Fc$_\epsilon$R consists of a single 45-kilodalton (45kd) to 50kd polypeptide specific for IgE (5) and that in its solubilized form the receptor is functionally multivalent (6).

In this report we continue the study of the murine B cell Fc$_\epsilon$R by investigating its structure with chemical cross-linking reagents. The studies indicate that the Fc$_\epsilon$R exhibits a spatial relationship with immunoglobulin (Ig) on the B cell surface in that a relatively high percentage of the B cell Fc$_\epsilon$R becomes consistently cross-linked to surface Ig (sIg). This interaction does not appear to be maintained if the cell membranes are disrupted by nonionic detergent in that cell extracts do not exhibit a similar cross-linking pattern. Further studies indicate that the B cell receptor does not appear to contain membane-buried components analogous to the β and γ subunits of the well-characterized high-affinity mast cell/basophil Fc$_\epsilon$R (7, 8). Finally, we report that the murine B cell Fc$_\epsilon$R in its solubilized state forms discrete complexes that may provide an explanation for the previously described multivalency (6).

MATERIALS AND METHODS

Antibodies and immunosorbents. Rat IgE was purified from IR 162 ascites by the method described by Isersky *et al.* (9). Where indicated, it was coupled, as described (5), to Affi-Gel-10 (Affi; Bio-Rad Laboratories, Richmond, CA). Purified mouse IgE specific for DNP, from the hybridoma H1-DNP-ϵ-26 (10), and polyclonal antisera directed against mouse IgM and mouse Ig were generously provided by Dr. K. Ishizaka. Bovine serum albumin (BSA) was reacted with trinitrobenzenesulfonate (TNP) to give TNP[18]-BSA (11), and the resulting material was coupled to Affi at a concentration of 2 mg/ml Affi (TNP-BSA-Affi). The anti-mouse Ig was used as either F(ab′)$_2$ or intact preparations; the anti-IgM was coupled to Affi. Monoclonal anti-IgD was a gift from Becton-Dickinson Laboratories (Sunnyvale, CA). Monoclonal antibody specific for the Fab′ region of the rat IgE, described by Conrad *et al.* (12), was used. Rabbit anti-mouse IgE (RAME) was affinity purified on a mouse IgE column. For use in some of the cross-linking experiments with rat basophilic leukemia (RBL) cells, rat IgE was amidinated with the use of HCl-methyl acetimidate (Pierce Chemical, Rockford, IL) by employing the method of Wofsy and Singer (13) as modified by Holowka *et al.* (7). Amidinated IgE could not be used with murine B cells (see *Results*).

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[2] Salary support to W. T. L. is from the National Institutes of Health Training Grant AI 07247.
[3] Abbreviations used in this paper: Fc$_\epsilon$R, receptor for the Fc portion of immunoglobulin E; DTSSP, 3,3′-dithio*bis*(sulfosuccinimidyl propionate); BS[3], *bis*(sulfosuccinimidyl) suberate; RBL, rat basophilic leukemia; sIg, surface immunoglobulin; kd, kilodalton; RAME, rabbit anti-mouse IgE; NP-40, Nonidet P-40; PMSF, phenylmethylsulfonylfluoride; lysis buffer, PBS containing 0.5% NP-40 and 1 mM PMSF; PO$_4$ buffer, 0.106 M sodium phosphate, pH 7.4; PO$_4$ lysis buffer, PO$_4$ buffer containing 0.5% NP-40 and 1 mM PMSF; tris-saline, 0.025 M tris, 0.14 M NaCl, pH 7.4; Staph a; protein A-bearing *Staphylococcus aureus*; 2-ME, 2-mercaptoethanol; Affi, Affi-Gel-10.

Cells. RBL cells from the 2H3 subline (14) were maintained in Eagle's MEM containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml gentamicin. Murine B cell preparations were made by using the spleens from BALB/c mice (Charles River Laboratories, Wilmington, MA) that were injected subcutaneously 12 to 16 days previously with 800 *Nippostrongylus brasiliensis* larvae. The purification of the B cells was as described (5). The resulting cell preparations were 98% viable and 75 to 80% sIg positive.

Isolation of the cell surface Fc,R and cross-linking methodology. Samples of RBL cells (2 to 4 × 10^7) or purified murine B cells (1 to 2 × 10^8) were suspended in 1 ml of phosphate-buffered saline (PBS) and were surface radiodinated with carrier-free ^{125}I (Amersham Searle Corp., Arlington Heights, IL) with the use of the lactoperoxidase system as described (15). Either before or after cross-linking, the cells were solubilized with one of the following two detergent buffer systems: a) lysis buffer (PBS containing 0.5% Nonidet P-40 [NP-40] and protease inhibitors as described (5) (the 50 mM ε-aminocaproic acid also served to quench cross-linking reactions—see below); b) PO_4 lysis buffer (0.106 M sodium phosphate, pH 7.4 [PO_4 buffer] containing 0.5% NP-40 and 1 mM phenylmethylsulfonylfluoride [PMSF]). Chemical cross-linking was performed at the a) intact cell, b) solubilized cell extract, or c) purified-receptor state. In the first two instances, cells were incubated for 2 hr with excess IgE (either normal or amidinated). After washing with PO_4 buffer, the cells were either solubilized with 1 ml PO_4 lysis buffer or immediately resuspended to 1 ml in PO_4 buffer and incubated with 3 to 10 mM 3,3'-dithio*bis*(sulfosuccinimidyl propionate) (DTSSP) or *bis*-(sulfosuccinimidyl) suberate (BS^3) for 30 min at 0°C. These are homobifunctional, cross-linking reagents that are cleavable or noncleavable, respectively, by reduction of an internal disulphide bond. Both DTSSP and BS^3 were originally received as gifts from Mr. E. Fujimoto and later were purchased from Pierce Chemical Co. Dose-response and kinetic studies performed on the RBL cell established the concentration (3 to 10 mM of cross-linker and the time of cross-linking (30 min at 0°C) as optimal in this system. Quenching of the intact cell cross-linking reactions was accomplished by washing the cells with tris-saline (0.025 M tris, 0.14 M NaCl, pH 7.4), followed by solubilization in lysis buffer and immunoprecipitation with monoclonal anti-rat IgE and protein A-bearing *Staphylococcus aureus* (Staph a). The quenching of receptor cross-linking in the fluid phase was accomplished by the addition of lysis buffer supplemented with 50 mM arginine followed by immunoprecipitation as above.

In some experiments, radiolabeled cells were first solubilized in either of the detergent systems and the clarified lysate was rotated with rat IgE-Affi for 2 hr to overnight. The immunosorbent was then placed in a small column and washed with approximately 100 bed volumes of the corresponding wash buffer (PBS containing 0.5% NP-40). The bound material was eluted by using the protocol of Kulczycki (16). Elution was accomplished with 0.2 N acetic acid containing 0.5% NP-40. NP-40-containing samples were immediately neutralized with 1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)/0.5% NP-40. Cross-linking of affinity-purified receptor preparations was performed by addition of the cross-linking reagent to the desired concentration for 30 min at 0°C, followed by quenching the cross-linking reaction with lysis buffer containing 50 mM arginine.

Double cross-linking experiments. Rat IgE was incubated with surface-radiolabeled B cells or RBL cells as above. The cells were washed three times with PO_4 buffer and incubated with 5 mM DTSSP for 30 min on ice, followed by an additional 30-min period in which DTSSP was again added to increase the concentration of DTSSP to 10 mM. The reaction was quenched by washing with tris-saline, reequilibrated with PO_4 buffer by washing two times, and solubilized with PO_4 lysis buffer. The clarified lysate was divided into two parts and incubated with or without 5 mM BS^3 for 30 min on ice. The reaction was quenched with lysis buffer, and immunoprecipitation with monoclonal anti-rat IgE was performed as above.

Biosynthetic labeling. RBL cells were grown in 75-cm^2 flasks to confluency and subsequently were cultured overnight in RPMI 1640 medium containing 10% dialyzed FBS and 16 μCi/ml [^3H] L-amino acid mixture (ICN Radiochemicals, Irvine, CA). The RPMI media was prepared from a select-amine kit (GIBCO, Grand Island, NY) and lacked the following amino acids: isoleucine, leucine, valine, phenylalanine, serine, and tyrosine. Anti-DNP mouse IgE (10 μg/ml) was added, and after 90 min at 37°C, the cells (5 × 10^6) were collected, washed two times with PO_4 buffer, lysed with PO_4 lysis buffer, and cross-linked with 5 mM DTSSP as described above. After quenching, the sample was mixed for 30 min with BSA-Affi, and the supernatant plus one wash was added to TNP-BSA-Affi. After mixing for 45 min (4°C), the adsorbant was added to a disposable column and washed with 100 bed volumes of PBS/NP-40, and the bound material was eluted with 0.1 M DNP in PBS/NP-40, pH 7.4. After a clearing precipitation with human IgE (8 μg) and 10 μg of affinity-purified rabbit anti-human IgE, the receptor complex was precipitated with affinity-purified RAME and Staph a.

Analysis of membrane Ig. A sample of surface-radiolabeled B cells was solubilized, and the sIg was immunoprecipitated with rabbit anti-mouse Ig and Staph a. The remaining cells were incubated for 2 hr with rat IgE and were subsequently treated with 5 mM DTSSP either before or after solubilization with PO_4 lysis-buffer. The IgE/Fc,R complex was immunoprecipitated as above, and all precipitates were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) after reduction of disulfide bonds. The respective x-ray films (see below) were analyzed by densitometry, and the contribution of individual components was determined by cutting and weighing the corresponding bands. By this procedure, 95% of the radioactivity in the anti-Ig precipitate was found in the sIg bands (μ, δ, and L chains), and this correction allowed calculation of the total cpm in sIg in the respective B cell sample.

In other experiments, the percentage of Fc,R cross-linked to sIg was determined. Radiolabeled B cells (4 × 10^6) were incubated with anti-DNP mouse IgE for 2 hr and after washing, the intact cells were treated with DTSSP as above, then were solubilized with lysis buffer, and the Fc,R complex was isolated by affinity binding to a TNP-BSA-Affi column. Elution was accomplished with 0.1 M DNP as described above. The eluted fractions were equally divided; the sIg was removed from one sample by repeated (three times) immunoprecipitation with anti-μ, anti-δ, and Staph a; the control sample received Staph a only. The IgE/Fc,R complex was then immunoprecipitated by adding RAME and Staph a, and the washed immunoprecipitates were analyzed by SDS-PAGE. Densitometric tracings were obtained and analyzed as above except that in these experiments the percentage of cpm attributable to the Fc,R after sIg removal was determined.

SDS-PAGE. Samples isolated by affinity chromatography were dialyzed against distilled water and lyophylized in a speed vac (Savant Instruments, Hicksville, NY). Excess detergent was removed by acetone extraction (17), and the samples were prepared for SDS-PAGE by boiling for 90 sec in sample buffer. Staph a-immunoprecipitated samples were treated similarly except that the Staph a was removed by centrifugation before application to the gel. Where applicable, samples were reduced by the addition of 5% 2-mercaptoethanol (2-ME). SDS-PAGE was performed with either slab or tube gels as indicated. Unless otherwise indicated slab gels were 1.5-mm 8 to 18% acrylamide gradient slab gels prepared and run as described (18). Molecular weight standards were coelectrophoresed with each run. The stained gels were dried by using a Bio-Rad model 224 gel dryer, and radioautography was performed at −70°C by using Kodak XAR-5 x-ray film and Dupont lightening plus intensifying screens (DuPont de Nemours Co., Wilmington, DE). Development was performed as described (18), and autoradiograms were scanned with a model GS-300 gel scanner (Hoeffler Scientific Instruments, San Francisco, CA). Tritium-containing samples were run on 15% acrylamide tube gels. Following the run, the gels were sliced into 2-mm slices and placed in a scintillation vial, and 0.5 ml NCS tissue solubilizer was added. After two hr at 55°C, 10 ml of Biocount (Research Products, Inc., Mount Prospect, IL) and 50 μl of acetic acid were added, and the samples were counted in a Packard Model 300C liquid scintillation counter (Packard Instruments Co., Downers Grove, IL).

RESULTS

Analysis of the cross-linking ability of DTSSP and BS^3. The initial objective of these studies was to examine the murine lymphocyte Fc,R for the presence of receptor-associated polypeptides other than the 49kd component previously described. Before doing so, we first tested the *N*-hydroxy-succinimidyl (NHS)-type cross-linking reagents, DTSSP and BS^3, by using the high-affinity Fc,R from RBL cells. Surface labeled RBL cells were divided into two equal portions, incubated with amidinated rat IgE for 2 hr, and washed to remove unbound IgE. One portion was resuspended in PO_4 buffer and cross-linked with 5 mM DTSSP and subsequently quenched with lysis buffer containing 50 mM arginine. The second portion was solubilized in PO_4 lysis buffer, clarified by centrifugation, and the cell lysate was cross-linked similarly to the intact cells with 5 mM DTSSP. The IgE/Fc,R complexes were then immunoprecipitated and examined by

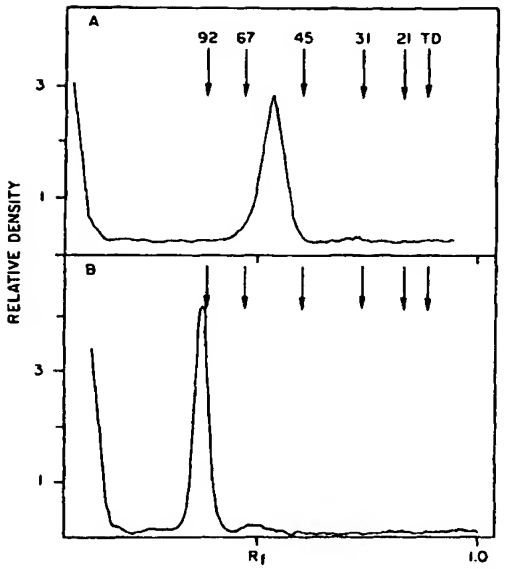

Figure 1. Confirmation of membrane impermeant nature of cross-linking reagents. Amidinated rat IgE was incubated with surface-labeled RBL cells (4×10^7); after washing, cells were treated with 5 mM DTSSP either (*A*) before or (*B*) after membrane solubilization with NP-40. IgE/Fc,R complexes were then isolated from detergent lysates by specific immunoprecipitation and were analyzed by SDS-PAGE slab gels. Migration position of m.w. standards (in kilodaltons) co-electrophoresed on the same gel is shown by arrows.

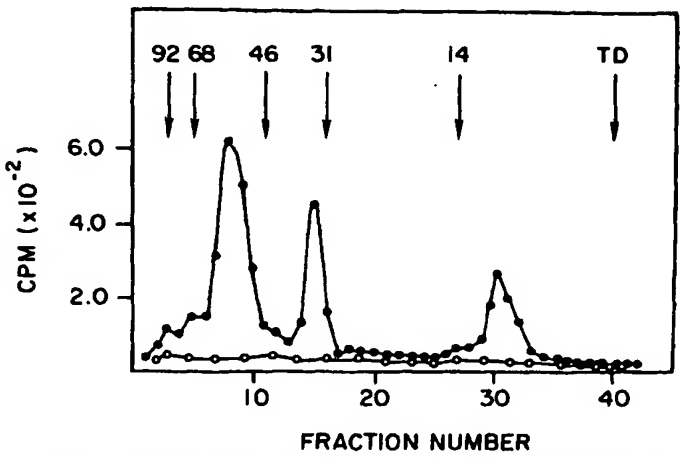

Figure 2. Determination of cross-linked RBL Fc,R components. RBL cells, grown as adherent monolayers, were cultured overnight in the presence of [^{3}H]-amino acid mixture; after 90-min incubation at 37°C with a saturating amount of anti-DNP mouse IgE, cells (5×10^6) were harvested, washed free of unbound IgE, and after membrane solubilization, treated with 5 mM DTSSP. IgE/Fc,R complexes were then isolated by affinity chromatography on TNP-BSA-Affi followed by immunoprecipitation of DNP eluate. Samples were analyzed on a 15% tube gel, and radioactivity was measured in gel slices by liquid scintillation counting. (●—●), RAME and Staph a immunoprecipitate; (○—○), control immunoprecipitate with use of normal rabbit IgG and Staph a. R_f of m.w. standards, run on a companion gel, is shown by arrows.

SDS-PAGE. Figure 1 shows that, in agreement with Staros (19), DTSSP is membrane impermeable. In similar experiments, BS^3 behaved identically. When cross-linking was performed on intact cells, only the ~58kd band previously shown to be the α subunit of the high-affinity Fc,R was present (Fig. 1*A*). However, when cross-linking was performed on the solubilized receptor, the apparent m.w. shifted upward to 100kd in this gel system (Fig. 1*B*). In the presence of 2-ME, the 100kd band is reduced to the ~58kd band, similar to that seen in Figure 1*A*. Holowka, Perez-Montfort, and co-workers (7, 8) have demonstrated that this higher m.w. form consists of a cross-linked complex containing the ligand binding α subunit and the β and γ subunits of the receptor. These associated subunits are not exposed at the cell surface and thus are not susceptible to surface iodination; however, they can be visualized by biosynthetic labeling with [^{3}H]-amino acids. Figure 2 demonstrates the presence of the β and γ components in the DTSSP cross-linked product. Biosynthetically labeled Fc,R was purified and analyzed by SDS-PAGE. In the presence of 2-ME, three peaks are seen with m.w. of ~58kd, 35kd, and 11kd, corresponding to the Fc,R α, β, and γ components, respectively. In the absence of 2-ME, the [^{3}H] radiolabel remained at the top of the gel (data not shown). Thus, DTSSP and BS^3 perform in a manner similar to the previously used imidates with the exception that they are membrane impermeable.

Cross-linking of IgE to the B cell Fc,R. Experiments with intact B cells were performed to determine if the B cell IgE/Fc,R complex would cross-link to other surface-exposed components. Surface-labeled B cells were incubated with rat IgE for 2 hr and after one wash with PO_4 buffer, the cells were treated with 5 mM DTSSP for 30 min at 0°C and subsequently were solubilized with lysis buffer. A second identical sample of labeled B cells was solubilized in lysis buffer immediately after washing. With both groups of cell lysates, the IgE/Fc,R complex was immunoprecipitated and analyzed by SDS-PAGE. Figure 3 demonstrates that in the absence of cross-linking, the 49kd component, previously described to be the B cell Fc,R, can be coprecipitated with IgE and anti-IgE. It should be noted that immunoprecipitation was allowed

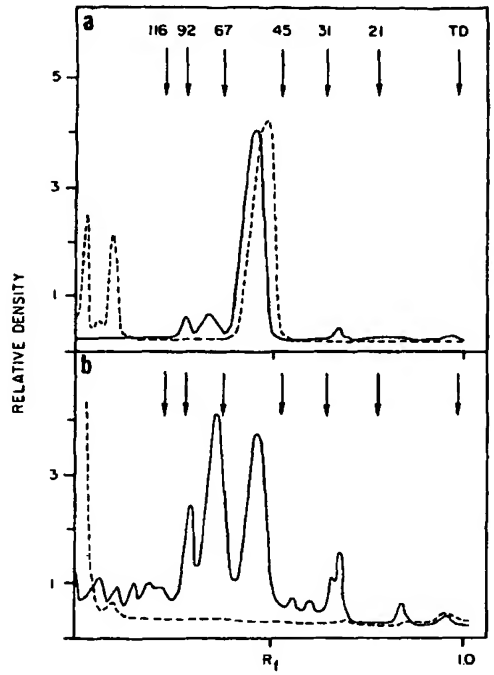

Figure 3. Analysis of immunoprecipitated B cell Fc,R. Purified B cells (2×10^6) were surface labeled and incubated with 1 mg IgE for 2 hr on ice. Washed cells were (*a*) solubilized, and IgE/Fc,R complexes were immediately immunoprecipitated or (*b*) intact cells were treated with DTSSP before solubilization with lysis buffer and specific immunoprecipitation. Densitometric scans of SDS-PAGE analyses are shown. (——) and (----), reduced and unreduced samples, respectively.

to occur immediately after clarification of the cell lysate to minimize dissociation. Figure 3*b* shows that if IgE is cross-linked to B cells with DTSSP before solubilization of the Fc,R, the 49kd band disappears and a single unresolved high m.w. band appears at the top of the gel. Upon disulfide reduction, four major bands are seen: one is the 49kd Fc,R, and the remaining three correspond to μ, δ, and L chains from B cell sIg. These bands have exactly the same relative migration as μ, δ, and L chains from specific immunoprecipitates performed as described (5) and, in addition, are not present if sIg is shed before cross-linking is performed (see Fig. 6).

To determine if the observed cross-linking would occur if the membrane was disrupted before the cross-linking procedure, we cross-linked rat IgE by DTSSP to the B cell Fc,R either before or after solubilization in PO_4 lysis buffer. As can be seen in Figure 4, if the B cell is solubilized before the cross-linker was administered, the isolated Fc,R sample contained very little sIg, indicating that any potential interaction between sIg and the Fc,R is abrogated by detergent solubilization.

We next attempted to define the relationship between the receptor and sIg by determining the percentage of sIg that would become cross-linked to the Fc,R and, conversely, the percentage of Fc,R that would cross-link to sIg. A sample of surface-labeled B cells was solubilized, the extract was immunoprecipitated with anti-mouse Ig, and the amount of radiolabeled sIg was determined. The remainder of the labeled B cell preparation was incubated with IgE, and the intact cells were treated with 5 mM DTSSP as in Figure 3*b*. After solubilization, the immunoprecipitated complex was examined by SDS-PAGE, and analysis of densitometric scans indicated that approximately 9% of the total sIg was cross-linked to the Fc,R (Table I). Because the number of sIg molecules per B cell is about one order of magnitude higher than the number of Fc,R molecules per B cell (3, 20), the data suggest that a relatively high percentage of the Fc,R molecules are becoming cross-linked to sIg. This suggestion was tested by the following experiment. Anti-DNP mouse IgE was cross-linked to intact B cells, and the IgE-containing complexes were isolated by affinity chromatography on a TNP-coated column (see *Materials and Methods*). The eluted material was divided into two equal portions; sIg was removed from one portion by successive immunoprecipitation with anti-μ and anti-δ, and the IgE/Fc,R complex was immunoprecipitated from both portions with the use of RAME and Staph a. Comparison of the Fc,R bands in the SDS-PAGE analysis of these samples indicated that ~50 to 60% of the receptor was removed by the anti-sIg immunoprecipitation treatment (Table I). In separate control experiments, the isolated sIg/IgE/Fc,R complexes were not removed by rabbit IgG Affi columns, confirming that the anti-Ig treatments were not removing any Fc,R by an Fc-mediated process.

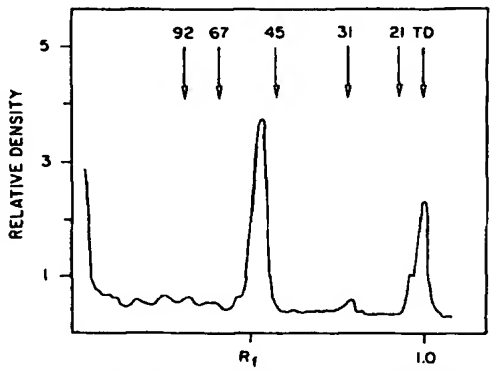

Figure 4. Cross-linking of B cell Fc,R after solubilization. Conditions were same as Figure 3*b* except that cells were solubilized in PO_4 lysis buffer before treating with DTSSP. SDS-PAGE analysis of reduced IgE/Fc,R immunoprecipitate is shown.

TABLE I
Analysis of the cross-linked complex

	Percent Cross-linked to Fc,R[a]			Percent Fc,R Cross-linked to sIg[b]
	μ	δ	sIg	
Intact cells[c]	10.5	8.5	9.0	59.5
Solubilized cells	0.5	0.3	0.4	ND[d]

[a] Values determined by weighing individual peaks from SDS-PAGE densitometric scans of IgE-Fc,R-specific or mouse sIg-specific immunoprecipitates, calculating cpm attributable to each component, and expressing values as sIg in IgE-Fc,R immunoprecipitates as a percent of total sIg.
[b] Values determined as above with percent of Fc,R cross-linked to sIg expressed as: 100 − percent of receptor remaining after removal of sIg by anti-IgM and anti-IgD.
[c] Values given represent the mean of two experiments.
[d] Not done.

Receptor-associated protein(s) in B cells. We next wanted to determine if components analogous to the β- and γ-chains of the RBL Fc,R are present in the B cell systems. The experiment described by Figure 1, namely isolation of the high-affinity Fc,R complex after solubilizaton and cross-linking of receptor bound to amidinated IgE, is analogous to the method employed by Holowka *et al.* (7), who first demonstrated the existence of the β component. In preliminary experiments it was determined that amidinated IgE did not interact well with the B cell Fc,R, preventing its use for the isolation of B cell Fc,R. This observation prompted the development of alternative means of cross-linking to determine the presence of components buried in the membrane. One such method takes advantage of the membrane impermeability of the two cross-linkers, DTSSP and BS^3. Because the putative β and γ components of the B cell Fc,R would be buried within the membrane, the use of a double cross-linking method should demonstrate its presence. Thus, purified B cells or RBL cells were incubated with rat IgE. After washing in PO_4 buffer to remove unbound IgE, the cells were treated with excess DTSSP to cross-link IgE to the receptor. The cells were then solubilized with PO_4 lysis buffer, and the clarified lysate was treated with BS^3, followed by immunoprecipitation and SDS-PAGE in the presence of 2-ME. It was anticipated that reduction would cleave the DTSSP, causing separation of those components cross-linked on the cell surface, specifically IgE from the surface accessible part of the receptor, and that any components that were associated within the membrane would appear as cross-linked products because of the noncleavable characteristics of BS^3. That this is true with the RBL cell Fc,R is shown in Figure 5, where it can be seen that when both DTSSP and BS^3 were used in this manner, the high m.w. band consisting of cross-linked α, β, and γ components was seen. Interestingly, when the murine B cells were treated in such a manner, there was no difference between the single or double cross-linked products, i.e., no appearance of a high m.w. band analogous to the RBL situation (data not shown). However,

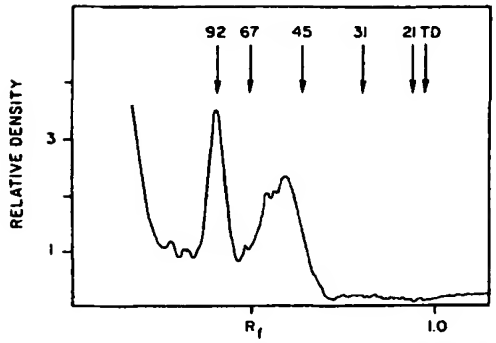

Figure 5. Double cross-linking of RBL Fc,R. Surface-labeled RBL cells were saturated with rat IgE. Washed cells were treated with 10 mM DTSSP over 1-hr period; after solubilization, cells were divided into two portions. One group was treated with 5 mM BS^3 for 30 min on ice; second group received buffer only. SDS-PAGE analysis of reduced sample treated with both cross-linking reagents is shown; sample treated with only DTSSP gave gel pattern similar to that shown in Figure 1*A*.

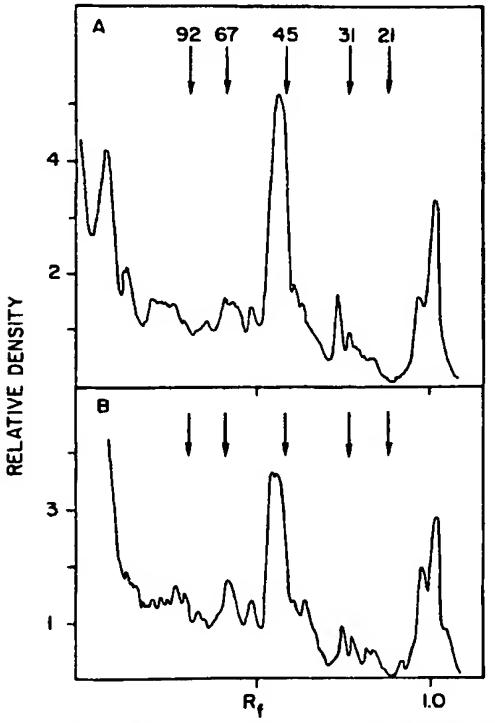

Figure 6. Double cross-linking of B cell Fc,R. B cells were first stripped of sIg by treatment with $F(ab')_2$ (see *Results*). Cells were then surface labeled, saturated with rat IgE, and treated either with (*A*) DTSSP or (*B*) with both DTSSP and BS^3 as in Figure 5. SDS-PAGE analysis of isolated IgE/Fc, precipitates, after reduction, is shown.

because of the presence of μ- and δ-chains from B cell sIg (see above) in the region where the cross-linked product might be seen, it is possible that these proteins obscured visualizaton of the cross-linked product. Therefore, the experiment was repeated after first causing the shedding and/or internalization of sIg by incubation of the cells with a predetermined optimal amount of $F(ab')_2$ anti-mouse Ig for 30 min at 37°C. Under these conditions, 5% of the B cell preparation remained sIg+ as compared with 78% sIg+ in the untreated control as determined by staining with fluorescein isothiocyanate (FITC)-anti-mouse Ig. Figure 6 shows the double cross-linking experiment performed with these cells, and again, there appears to be little difference between the single or double cross-linked products with respect to the 49kd Fc,R entity.

Cross-linking experiments with the purified Fc,R. Previous work has demonstrated that both the RBL Fc,R (21) and the B cell Fc,R (2, 5) can be purified to near homogeneity with respect to the surface-labeled components by affinity chromatography on IgE-coated adsorbents. Thus, we next attempted to confirm the results of the double cross-linking study by performing cross-linking experiments on affinity-purified Fc,R preparations from RBL cells and B cells. The isolation of the surface-radiolabeled B cell Fc,R via IgE conjugated to a solid matrix (IgE-Affi) has been shown to isolate predominantly a single 49kd band by SDS-PAGE (5). Isolation of the RBL Fc,R by similar methods reveals only the ~58K surface labeled α component. It is not clear from previous work (see *Discussion*) whether the β and γ subunits of the receptor remain associated with the α-IgE complex under the acid elution conditions used. Thus, RBL cells were surface labeled, and the radiodinated α component was eluted from an IgE-Affi column, immediately neutralized, and treated with 10 mM DTSSP (final concentration) as described in *Materials and Methods*. The cross-linked material was examined by SDS-PAGE, and as can be seen in Figure 7, very little (<10%) of the α component was cross-linked to form the ~100kd α, β, γ complex under these conditions. As a control, the same experiment as described in Figure 1*B* was performed except that the cells were solubilized in the acetic acid/HEPES/NP-40 mixture that was used in the acid elution protocol. Results exactly analogous to those shown in Figure 1*B* were obtained, indicating that this buffer system can support cross-linking. Thus, these results indicate that the majority of β and/or γ is dissociated from the α component by the acid elution purification procedure and, in addition, indicate that there is no association between α components under these conditions to yield higher m.w. oligomers. In addition, these results support published reports that demonstrated monovalency of the high-affinity Fc,R (22).

Figure 8 demonstrates the results of cross-linking the B cell Fc,R in the NP-40-containing system. It is clear that the low-affinity Fc,R is susceptible to cross-linking in the NP-40 system but that, in accord with the double cross-linking experiments, there is no band equivalent to the 100kd RBL Fc,R complex. Rather, the 49kd compo-

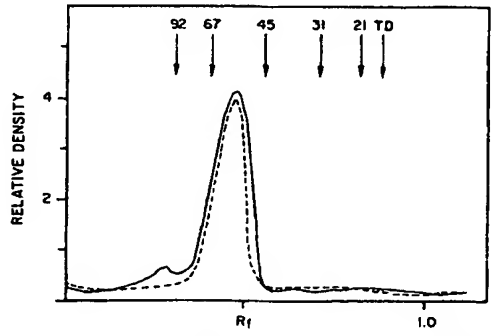

Figure 7. Cross-linking of RBL Fc,R after affinity purification. Fc,R from 4×10^7 surface-labeled RBL cells was isolated by affinity chromatography on IgE-Affi. Purified Fc,R was then treated with 3 mM DTSSP and subsequently analyzed by SDS-PAGE. (-----) reduced and (——) unreduced samples, respectively.

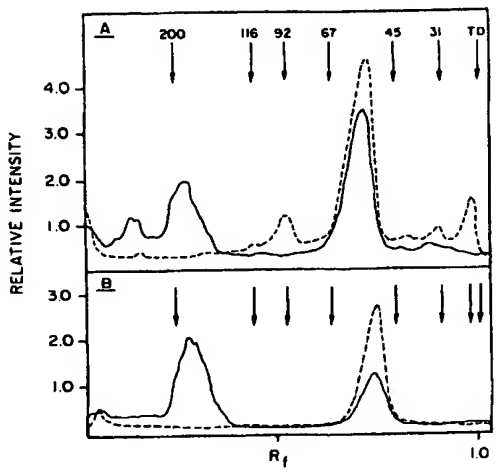

Figure 8. Cross-linking of B cell Fc,R after affinity purification. B cell Fc,R from 4 × 10^6 surface-labeled B cells was isolated by affinity chromatography on IgE-Affi. One-half of initial purified Fc,R preparation was subjected to a second cycle of affinity chromatography on IgE-Affi. Both Fc,R preparations were then treated with 3 mM DTSSP and examined by SDS-PAGE. (*A*) Fc,R preparation after one cycle on IgE-Affi and (*B*) after two-stage IgE-Affi purification procedure. (----) reduced and (——) unreduced samples, respectively.

nent becomes cross-linked to form two higher m.w. bands, a large peak at 160kd and a small peak above the 200kd marker. Upon reduction, the majority of the radiolabel migrates as the 49kd Fc,R. Successive rebinding to IgE adsorbents with subsequent elution has been found to be an effective way to further purify Fc receptors to near homogeneity, even with biosynthetically labeled preparations (16). As can be seen in Figure 8*A*, the Fc,R preparation contains additional surface labeled material, the most prominent of which is the 84kd band obtained under reducing conditions. Thus, the B cell Fc,R was further purified by a second cycle of binding and elution with IgE-Affi, and the final eluate was cross-linked with DTSSP. This highly purified Fc,R remains susceptible to cross-linking (Fig. 8*B*); the majority of the Fc,R now migrates as a 160kd component.

DISCUSSION

To facilitate the continued comparisons of the high- and low-affinity Fc,R, we undertook the cross-linking studies outlined in this report. We first sought to test BS^3 and DTSSP in our system, using the high-affinity Fc,R on RBL cells. As mentioned previously, the surface-exposed IgE-binding part of the high-affinity Fc,R (α) is known to be associated with two other components that are not thought to be accessible to the cell surface: β, a 35kd nonglycosylated polypeptide (7), and γ, a 20kd disulfide-linked dimer that is also buried in the membrane (8). When intact cells were used, IgE was cross-linked to the α component in efficiencies ranging as high as 80% (data not shown). Amidination of the IgE largely blocked the IgE-α cross-linking, however; when intact cells were used, cross-linking of α to β and γ was not seen (Fig. 1*A*). Solubilization of the cells before the addition of cross-linker allowed efficient cross-linking of α to a higher m.w. form (Fig. 1*B*), which was shown by biosynthetic labeling studies to contain only the three receptor components (Fig. 2). The m.w. of the three components is in reasonable agreement with previously published m.w. estimates of α (the high degree of glycosylaton makes the m.w. for α dependent on the gel system used (23)), β, and γ (7, 8). Vectorial labeling techniques and protease digestion studies (24) have previously indicated that β and γ were not exposed to the cell surface; thus, the results herein support this notion while confirming the membrane impermeant nature of DTSSP and BS^3.

We next sought to use these cross-linking reagents in the murine B cell Fc,R system, with the overall objective of comparison of the Fc,R in the two systems. First, surface cross-linking events were investigated and it was found that IgE could be cross-linked to the Fc,R (Fig. 3*b*) in that all of the 49kd Fc,R component was incorporated into a high m.w. complex at the top of the gel. However, a surprise was that some sIg also became cross-linked to the IgE/Fc,R complex. Interestingly, this laboratory previously reported that some sIg co-isolates with the Fc,R on IgE Sepharose (5); however, this sIg was not present if the affinity adsorbent was IgE-Affi, possibly because the spacer group on the IgE-Affi allows very efficient washing. Under these cross-linking conditions, approximately 10% of the total sIg is found in the final cross-linked product, suggesting that the sIg/IgE-Fc,R cross-linking is a relatively common phenomenon (Table I). Depletion of the sIg in the purified complexes indicated that about 60% of the IgE-Fc,R was becoming cross-linked to sIg.

The association of various cellular macromolecules with membrane Ig has been reported (25, 26). Indeed, Koch and Haustein (26) demonstrated a product of similar m.w. to the Fc,R that co-isolated with sIg after cross-linking studies. However, their observations that the cross-linking occurred only with sIgM is not borne out in this study. The interaction between IgE-Fc,R and sIg is apparently not maintained if the B cell membrane is disrupted before cross-linking; co-isolation of sIg is then almost completely abrogated (Fig. 4). In this light, Dickler (see Reference 27 for review) has described an interaction between the antigen receptor (sIg) and FcγR on B cells; interestingly, it was found that there is a requirement for ligand occupancy by both types of receptor in order for this interaction (co-capping) to occur (28). With the Fc,R/IgE system on B cells, shedding or internalization of sIg as induced by $F(ab')_2$ anti-mouse Ig does not decrease the percentage of cells that are Fc,R+ as determined by rosette analysis (data not shown). At this point, we cannot determine the relevance of this interaction to receptor or B cell function; however, experiments are in progress to test the effect of ligand (IgE) occupation on the Fc,R-sIg association in this system and to determine if quantitatively the number of Fc,R molecules per cell decreases when sIg is shed from the cells.

The ability to cross-link IgE to the Fc,R and immunoprecipitate by anti-IgE allows the investigation of the subunit structure of the low-affinity receptor. Recently, Finbloom and Metzger (29) demonstrated the presence of a 35kd component that becomes cross-linked with the low-affinity Fc,R on rat macrophages and suggested that this component is analogous to the β component found as part of the high-affinity RBL Fc,R. In light of this observation, they postulated that the Ig binding protein (i.e., Fc receptor) for various Ig was associated with a 35kd β-like component buried in the membrane. To investigate this possibility with regard to the murine B cell

Fc,R, we took advantage of the membrane impermeability of the cross-linking reagents DTSSP and BS^3. By employing DTSSP with intact B cells, we anticipated that the lysyl and arginyl residues available to cross-link IgE to the Fc,R would be completely reacted with the DTSSP reagent. Solubilization of the cells and the cross-linking with BS^3 would allow intramembrane-associated complexes to become available for covalent coupling that would be unaffected by reducton with 2-ME. This approach was first tested by cross-linking the high-affinity Fc,R complex of RBL cells. If both cross-linking reagents are employed, upon reduction and separation of the component from IgE, there is a shift to the higher m.w. form that was shown to be the α, β, γ, complex (Fig. 4). The double cross-linking experiment was performed on murine B cells both with (Fig. 6) and without first removing sIg by shedding. In both situations, the 49kd Fc,R is present and there is no evidence for a cross-linked complex analogous to the RBL, α, β, γ complex. Similar experiments to those reported in this study were performed on the 8866 human B lymphoblastoid Fc,R, yielding the same conclusion with respect to β/γ analogues (Peterson and Conrad, unpublished observations). Thus, our results would tend to argue against the presence of a receptor-associated β/γ analogue in the B cell system and would suggest that the B cell Fc,R is structurally dissimilar to both the high-affinity mast cell/basophil receptor and the low-affinity macrophage Fc,R. However, some precautions must be mentioned. There is an increased amount of material that does not enter the gel when the double cross-linking system is used with both RBL cells and B cells (Figs. 5, 6). The most likely explanation is that there is still a certain amount of cross-linking of receptor to IgE via BS^3 because the DTSSP treatment did not saturate all of the reactive sites. In addition, there exists the possibility that there is a very high m.w. nonsurface-labeled component that cross-links to the receptor and forms too large a complex to enter the gel. Another point is that the DTSSP used to form the receptor-IgE cross-links depletes those same groups that are involved in cross-linking the binding protein to the associated protein. However, it is clear that the double cross-linking methodology does work with the RBL cell, although with a lower efficiency than with the single cross-linking reagent (Fig. 1 *vs* Fig. 5).

The cross-linking of the Fc,R after affinity isolation provides some interesting information. With the high-affinity Fc,R on RBL cells, purification by the extensive washing and acid elution conditions used herein evidently caused extensive dissociation of the α, β, γ receptor complex. Only a relatively small amount of cross-linking to the ~100kd complex is seen after affinity purification (Fig. 7). The stability of the α, β, γ complex is known to be enhanced by the maintenance of a critical phospholipid/detergent ratio (30), and in separate experiments (Lee and Conrad, unpublished observations) the addition of phospholipids to the isolation system gave definite improvement to the results seen in Figure 7 in that up to 40% of the α was cross-linked to the 100kd α, β, γ complex (data not shown). There are some data suggesting that at least the β component can be isolated by repetitive affinity chromatography by using acid elution conditions similar to those used in this study (31); however, cross-linking reagents were not used in those studies, and thus, efficiency of isolation cannot be determined. In these experiments there was no evidence for α-α cross-linking, in agreement with the monovalency determination of this Fc,R (22).

The B cell Fc,R does clearly cross-link to defined higher m.w. components in these studies (Fig. 8). No component indicative to an α, β, γ complex is seen, and preliminary studies indicate that inclusion of phospholipid in the detergent systems does not change this result. The purified B cell Fc,R cross-links to a component that migrates as a 160kd component and, to a lesser extent, to a component that migrates slightly ahead of the 200kd marker. The cross-linking to form the 160kd component occurs even after a second cycle of affinity isolation of IgE-Affi. At this stage, the Fc,R is highly purified; similar studies have demonstrated that biosynthetically labeled Fc receptors are purified to near homogeneity by this repetitive affinity chromatography procedure (16). Thus, the possibility that the 160kd component is the result of the 49kd Fc,R becoming cross-linked to unlabeled or weakly labeled additional components seems unlikely; however, this possibility is at present being further investigated with regard to the one-stage purified Fc,R and the >200kd cross-linked product seen in Figure 8*A*. It is tempting to speculate that the 160kd component is a trimer of the 49kd Fc,R. In an earlier study, the murine B cell Fc,R was shown to be multivalent in detergent solution (6). Those data were most consistent with a divalent mode; however, because of dissociation, divalency was only an approximation. Assuming that the cross-linked product does represent the multivalent receptor, it can be concluded that the cross-linking between the different Fc,R components is occurring at sites that would be exposed on the surface of the cell, because essentially the same amount of 49kd receptor is recovered when either single or double cross-linking experiments (Fig. 6) are performed.

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REFERENCES

1. **Gonzalez-Molina, A., and H. L. Spiegelberg.** 1977. A subpopulation of normal human peripheral B lymphocytes that bind IgE. *J. Clin. Invest. 59:616.*
2. **Spiegelberg, H. L.** 1981. Lymphocytes bearing Fc receptors for IgE. *Immunol. Rev. 56:199.*
3. **Vander-Mallie, R., T. Ishizaka, and K. Ishizaka.** 1982. Lymphocytes bearing receptors for IgE. VIII. Affinity of mouse IgE for Fc,R on mouse B lymphocytes. *J. Immunol. 128:2306.*
4. **Yodoi, J., and K. Ishizaka.** 1979. Lymphocytes bearing Fc receptors for IgE. I. Presence of human and rat T lymphocytes with Fc,R receptors. *J. Immunol. 122:2577.*
5. **Conrad, D. H., and L. H. Peterson.** 1984. The murine lymphocyte receptor for IgE. I. Isolation and characterization of the murine B cell Fc, receptor and comparison with Fc, receptors from rat and human. *J. Immunol. 132:796.*
6. **Lee, W. T., and D. H. Conrad.** 1984. The murine lymphocyte receptor for IgE. II. Characterization of the multivalent nature of the B lymphocyte receptor for IgE. *J. Exp. Med. 159:1790.*
7. **Holowka, D., H. Hartmann, J. Kanellopoulos, and H. Metzger.** 1980. Association of the receptor for immunoglobulin E with an endogenous polypeptide on rat basophilic leukemia cells. *J. Recept.*

Res. 1:41.
8. **Perez-Montfort, R., J. P. Kinet, and H. Metzger.** 1983. A previously unrecognized subunit of the receptor for immunoglobulin E. *Biochemistry 22:5722.*
9. **Isersky, C., A. Kulczycki, Jr., and H. Metzger.** 1974. Isolation of IgE from reaginic rat serum. *J. Immunol. 112:1909.*
10. **Liu, F. T., J. W. Bohn, E. L. Ferry, H. Yamamoto, C. A. Molinaro, L. A. Sherman, N. R. Klinman, and D. H. Katz.** 1980. Monoclonal dinitrophenol specific murine IgE antibody preparation, isolation, and characterization. *J. Immunol. 124:2728.*
11. **Good, A. H., L. Wofsy, C. Henry, and J. Kimura.** 1980. Preparation of hapten-modified protein. In *Selected Methods in Cellular Immunology.* Edited by B. B. Mishell and S. M. Shiigi. W. H. Freeman and Co., San Francisco. P. 343.
12. **Conrad, D. H., E. Studer, J. Gesvasoni, and T. Mohanakumar.** 1983. Properties of two monoclonal antibodies directed against the Fc and Fab′ regions of rat IgE. *Int. Arch. Allergy Appl. Immunol. 70:352.*
13. **Wofsy, L., and S. J. Singer.** 1963. Effects of the amidination reaction on antibody activity and on the physical properties of some proteins. *Biochemistry 2:105.*
14. **Barsumian, E. L., C. Isersky, M. G. Petrino, and R. P. Siraganian.** 1981. IgE-induced histamine release from rat basophilic leukemia cell lines: isolation of releasing and non-releasing clones. *Eur. J. Immunol. 11:317.*
15. **Conrad, D. H., and A. Froese.** 1976. Characterization of the target cell receptor for IgE. II. Polyacrylamide gel analysis of the surface IgE receptor from normal rat mast cells and rat basophilic leukemia cells. *J. Immunol. 116:319.*
16. **Kulczycki, A., Jr.** 1983. Purification of Fc, receptors and Fc, receptors. In *Methods in Enzymology, Vol. 93.* Edited by S. P. Colowick and N. O. Kaplan. Academic Press, Inc., New York. P. 178.
17. **Hempstead, B. L., W. Parker, and A. Kulczycki, Jr.** 1979. Characterization of the IgE receptor isolated from human basophils. *J. Immunol. 123:2283.*
18. **Pecoud, A. R., S. Ruddy, and D. H. Conrad.** 1981. Functional and partial chemical characterization of the carbohydrate moieties of the IgE receptors on rat basophilic leukemia cells and rat mast cells. *J. Immunol. 126:1624.*
19. **Staros, J. V.** 1982. *N*-Hydroxysulfosuccinimide active esters: *bis* (*N*-hydroxysulfosuccinimide) esters of two dicarboxylic acids are hydrophilic, membrane-impermeant, protein cross-linkers. *Biochemistry 21:3950.*
20. **Rabellino, E., S. Colon, H. M. Grey, and E. R. Unanue.** 1971. Immunoglobulins on the surface of lymphocytes. I. Distribution and quantitation. *J. Exp. Med. 133:156.*
21. **Conrad, D. H., and A. Froese.** 1978. Characterization of the target cell receptor for IgE. III. Properties of the receptor isolated from rat basophilic leukemia cells by affinity chromatography. *J. Immunol. 120:429.*
22. **Kanellopoulos, J. M., T. Y. Liu, G. Poy, and H. Metzger.** 1980. Composition and subunit structure of the cell receptor for immunoglobulin E. *J. Biol. Chem. 255:9060.*
23. **Kulczycki, A., Jr., T. A. McNearney, and C. W. Parker.** 1976. The rat basophilic leukemia cell receptor for IgE. I. Characterization as a glycoprotein. *J. Immunol. 117:661.*
24. **Holowka, D., and H. Metzger.** 1982. Further characterization of the β-component of the receptor for immunoglobulin E. *Mol. Immunol. 19:219.*
25. **Rosenspire, A. J., and Y. S. Choi.** 1982. Relation between actin-associated proteins and membrane immunoglobulin in B cells. *Mol. Immunol. 19:1515.*
26. **Koch, N., and D. Haustein.** 1983. Association of surface IgM with two membrane proteins on murine B lymphocytes detected by chemical cross-linking. *Mol. Immunol. 20:33.*
27. **Dickler, H. B.** 1982. Interactions between receptors for antigen and receptors for antibody—a review. *Mol. Immunol. 19:1301.*
28. **Dickler, H. B., and M. T. Kubicek.** 1981. Interactions between lymphocyte membrane molecules. I. Interactions between B lymphocyte surface IgM and Fc IgG receptors requires ligand occupancy of both receptors. *J. Exp. Med. 153:1329.*
29. **Finbloom, D. S., and H. Metzger.** 1983. Isolation of crosslinked IgE-receptor complexes from rat macrophages. *J. Immunol. 130:1489.*
30. **Rivnay, B. S., A. Wank, G. Poy, and H. Metzger.** 1982. Phospholipids stabilize the interaction between alpha and beta subunits of the solubilized receptor for immunoglobulin E. *Biochemistry 21:6922.*
31. **Kulczycki, A., Jr., and C. W. Parker.** 1979. The cell surface receptor for immunoglobulin E. I. The use of repetitive affinity chromatography for the purification of a mammalian receptor. *J. Biol. Chem. 254:3187.*